FEBS Letters 579 (2005) 18–24 FEBS 29073

Structure of the *Bordetella trematum* LPS O-chain subunit

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Received 26 August 2004; revised 12 November 2004; accepted 12 November 2004

Available online 26 November 2004

Edited by Gerrit van Meer

Abstract Analysis of the O-chain subunit of the lipopolysaccharide (LPS, endotoxin) isolated from *Bordetella trematum*, a recently identified human pathogen, was undertaken. The polysaccharide (PS) moiety was shown to contain only two O-chain subunits, which differed in the anomeric bond of their first sugar. A trisaccharide fragment resulting from the cleavage of a FucNAc glycosidic bond was isolated after treatment of the PS with anhydrous HF. Nitrous deamination of the LPS led to the release of the following heptasaccharide corresponding to two trisaccharide subunits linked to an anhydromannitol residue. β-ManNAc3NAmA-(1-4)-β-ManNAc3NAmA-(1-3)-α-FucNAc-(1-4)-β-ManNAc3NAmA-(1-4)-β-ManNAc3NAmA-(1-3)-β-FucNAc-(1-6)-2,5-anhManol.

Keywords: *Bordetella trematum*; Endotoxin; Structure; MALDI; NMR

1. Introduction

Endotoxins are major components of the outer membrane of Gram-negative bacteria. They consist of complex mixtures of related lipopolysaccharides (LPS) of which the lipid region is covalently linked to a polysaccharide comprising a core region to which may be linked an O-chain having a variable number of subunits. In the latter case, the bacterial species is termed ''S-type'' because of the smooth aspect of its colonies and its LPS gives a ladder-like pattern of bands on SDS–PAGE gels. When the O-chain is absent, the colonies are rough and the bacteria termed ''R-type''. A third category, the ''S-R''-type, has an O-chain consisting of a single unit. This type of structure is the result of a deficiency in the O-chain polymerase enzyme [1].

B. pertussis is a Gram-negative bacterium responsible for whooping cough. Its LPSs have no O-chains, unlike those of most other species of the Bordetella [2,3]. Recent evidence has shown that *B. pertussis* lacks the genes for O-chain synthesis [4]. It has been reported that the pertussis endotoxin may be involved in the whooping-cough syndrome by inducing NO production and release, by tracheal epithelial cells, thereby poisoning and inactivating adjacent ciliated cells [5].

The polysaccharide (PS) moiety of the *B. pertussis* LPS is heterogeneous and gives two bands on SDS–PAGE analysis [6,7]. Band A, the more abundant, slower-migrating molecular species corresponds to an LPS with a dodecasaccharide core, and band B, to an LPS lacking the three distal (non-reducing end) sugars [7]. Band A LPS resembles an S-R-type LPS [7].

Eight species make up the Bordetella genus and the LPS structures of five of them have been described [2,3,8,9]. Most of the species are pulmonary tract pathogens. *Bordetella trematum*, on the other hand, has been found only in human ear and blood infections associated with immunodepression [10]. The LPSs of all of the Bordetella except *B. pertussis* and *B. trematum* give S-type profiles on SDS–PAGE.

In the present study, the *B. trematum* O-chain unit was separated from the polysaccharide moiety of the LPS and its structure analyzed by mass spectrometry and NMR.

2. Materials and methods

2.1. Bacterial strains and cultures

B. trematum strain CCUG 13902 was grown as described [8].The cells were killed in 2% phenol before harvesting.

2.2. LPS

LPS was extracted by the phenol–water method and purified as previously described [8].

2.3. Thin-layer chromatography

Chromatography was performed on aluminum-backed silica thin-layer chromatography (TLC) plates (Merck) and spots were visualized by charring (145 °C after spraying with 10% sulfuric acid in ethanol). The solvent was a mixture of isobutyric acid:M ammonium hydroxide (3:5, v/v) for polysaccharides and (5:3, v/v) for LPS [11].

2.4. Detergent-promoted hydrolysis

The LPSs (200 mg) were cleaved by hydrolysis in 20 mM Na acetate–acetic acid, pH 4.5 and 1% Na dodecylsulfate at 100 °C for 1 h at a concentration of 5 mg/ml [12].

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Abbreviations: COSY, correlation spectroscopy; TOCSY, total correlation spectroscopy; NOESY, nuclear overhauser enhancement spectroscopy; HMQC, heteronuclear multiple quantum coherence; HMBC, heteronuclear multiple bond connectivity; HSQC, heteronuclear single quantum correlation; ROESY, rotational nuclear overhauser effect spectroscopy; LPS, lipopolysaccharide; MALDI, matrix-assisted laser desorption/ionization mass spectrometry; FucNAc, 2-acetamido-2,6-deoxy-galactose; GlcN, glucosamine; ManNAc3NAcA, 2,3-diacetamido-2,3-dideoxy-mannuronic acid; ManNAc3NAmA, 2-acetamido-3-acetamidino-2,3-dideoxy-mannuronic acid

0014-5793/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2004.11.040

2.5. Purification of the polysaccharide moiety

After centrifugation, the supernatant containing the polysaccharides was lyophilized (110 mg), taken up in 2 ml of column buffer (0.05 M pyridine–acetate, pH 5), and chromatographed on a Sephadex G-50 column (45 × 1.6 cm). TLC indicated the presence of two polysaccharide peaks of different migration rate. Their respective fractions were pooled and lyophilized.

2.6. Solvolysis of the slower migrating polysaccharide fraction

Polysaccharide (50 mg) was dissolved in liquid anhydrous hydrogen fluoride (4 ml) and stirred at room temperature for 3 h [2]. HF was removed by a stream of nitrogen gas. The residue taken up in water was neutralized with dilute ammonia, de-ionized with Resin 101 (H^+) and AG 3-X4 (OH^-), reduced with $NaBH_4$, and chromatographed by Sephadex G-50 gel-filtration to yield a major fraction (30 mg) and some minor ones. The products were separated further on a C18 reverse-phase column (Aqua 250 × 9 mm, Phenomenex) in water with UV detection at 220 nm. A compound **1** and two compounds **2** and **3** were obtained and prepared for NMR analysis.

2.7. Nitrous deamination

Nitrous deamination of the LPS (5 mg/ml) was done as described previously [2,13]. This cleaved the LPS polysaccharide chain at the level of amino sugars to give a soluble oligosaccharide with a terminal anhydro sugar plus free anhydro sugars, and an insoluble fraction assumed to include the lipid A. The reaction mixture was centrifuged at 200 000 × *g* for 2 h to separate these two fractions. The supernatant was adjusted to pH 4–5 with M NaOH, taken to dryness under reduced pressure, redissolved in 15 ml water, and centrifuged (3000 × *g*, 10 min). The supernatant diluted with 40 ml water and dialyzed in a Diaflo cell under N_2 pressure with a UM05 membrane (Amicon Corp.) to remove the salt. It was then reduced with NaBD4 and purified on a Sephadex G 50 column as described for the PS fraction. The major fraction obtained was separated on a C18 reverse-phase column to give compounds **2**, **3** and **4**, which were then prepared for NMR analysis.

2.8. Gas chromatography

Hydrolysis was performed with 4 M CF_3CO_2H (110 °C, 3 h), monosaccharides were reduced and peracetylated to their alditol acetate derivatives and analyzed by gas chromatography (GC) on an Agilent 6850 chromatograph equipped with a DB-17 (30 m × 0.25 mm) fused-silica column using a temperature gradient of 180 °C (2 min) → 240 °C at 2 °C/min.

2.9. Mass spectrometry

MALDI/MS was carried out on a Perseptive Voyager-DE STR model (PE Biosystem, France) time-of-flight mass spectrometer (IBBMC, IFR 46, Orsay). Gentisic acid (2,5-dihydroxybenzoic acid from Sigma Chemical, St. Louis) 10 mM in a 0.1 M citric acid solution [14] was used as matrix. Dowex 50 (H^+)-decationized samples (0.5 μg/0.5 μl) were deposited on the target, covered with 0.5 μl of the matrix solution and dried. Analyte ions were desorbed from the matrix with pulses from a 337 nm nitrogen laser. Spectra were obtained in the negative-ion mode at 20 kV with an average of 128 pulses. The masses are average masses.

MALDI postsource decay (PSD) TOF MS experiments were performed to study fragmentation patterns of oligosaccharides. The samples were prepared as described above. The laser power used was the minimum necessary to obtain adequate fragmentation and the reflectron voltage was stepped down from 20 kV in five steps.

ESI/MS spectra were obtained using a Micromass Quattro spectrometer in 50% MeCN with 0.2% HCOOH at a flow rate of 15 μl/min with direct injection of the sample.

2.10. NMR spectroscopy

NMR spectra were recorded at 25 °C in D_2O on a Varian UNITY INOVA 500 instrument, using acetone as reference for proton (2.225 ppm) and carbon (31.5 ppm) spectra. Varian standard programs COSY, ROESY (mixing time of 400 ms), TOCSY (spinlock time 120 ms), HSQC, and gHMBC (long-range transfer delay 100 ms) were used with digital resolution in F2 dimension < 2 Hz/pt.

3. Results and discussion

3.1. Solvolysis of the polysaccharide

Preliminary analysis by mass spectrometry and SDS–PAGE having indicated similarities between the *B. pertussis* and the *B. trematum* polysaccharides, the structural analysis of the latter's distal region was undertaken in a way similar to that used for the *B. pertussis* PS [2]. This treatment had been shown to be useful for cleaving core sugar anomeric bonds but preserved those of diaminouronic sugars suggested to be present by the preliminary MS analysis. Trisaccharide 1 and monosaccharides 2 and 3, as well as minor products were obtained.

3.2. NMR analysis

A set of 2D NMR spectra of compound **1** (COSY, TOCSY, ROESY, HSQC, and HMBC) were recorded and completely assigned (Table 1, Fig. 1). The spectra corresponded to a reducing trisaccharide with α- and β-pyranose anomeric forms of FucNAc at the reducing end. Spin systems of two residues of β-2,3-diamino-2,3-dideoxymannuronic acid were also identified; the signals of these residues were broadened or split because of the attachment to α- or β-forms of FucN. The β-configuration of both ManNAcNA residues followed from the observation of the intraresidual NOEs between protons H-1 and H-3, H-1 and H-5. The sequence of the monosaccharides was determined from NOE (correlations between C1 and B4 protons, and between B1 and α- and β-A3 protons) and HMBC (proton to carbon correlations C1-B4, B1-αA3 and B1-βA3). The structural assignment was confirmed by comparison with the data for *Pseudomonas aeruginosa* O2 oligosaccharides [15], which had the same structure except for the replacement of an acetyl group on N-3 of the residue analogous to B in the *P. aeruginosa* products with an acetamidino group in the *B. trematum* product. As the absolute configuration of all monosaccharides in the *P. aeruginosa* product was found to be D and the NMR chemical shifts of all monosaccharides in the *B. trematum* products are virtually the same as in *P. aeruginosa*, absolute configurations of the monosaccharides in trisaccharide 1 and heptasaccharide 4 are assigned D.

The position of the acyl groups was deduced from HMBC data, where correlations from carbonyl carbons of acetyl or acetamidino groups to the ring protons at the attachment position of acylamino groups were observed. Two characteristic ^{13}C signals of C-1 of the amidino groups at ~167 ppm were present, giving HMBC correlations with H-3 of the β-ManNAc3NAmA residues B and C. Other protons at the positions of the amino group attachment in all monosaccharides gave correlations to acetate carbonyl signals around 175 ppm. The exceptional structural similarity of oligosaccharide 4 to the O-chains of *P. aeruginosa* O2 LPSs suggests immunological similarity, which remains to be investigated. However, the presence of an amidino group on every diaminouronic acid residue leads to an overall zero charge on the *B. trematum* LPS side chain. In *P. aeruginosa* each repeating unit has two negative and one positive charge, hence an overall negative charge. These differences will certainly influence their serological reactivity.

3.3. Nitrous deamination of the LPS

As expected from previous experiments performed on the *B. pertussis* LPS [2], the nitrous deamination of the whole

Table 1
NMR data for compounds **1** and **4**

Unit	Nucleus	Compound					
		1	**2**/NAc	**3**/NAm	**4**	**5**	**6**
α-A, 1	^{1}H	5.14	4.20	4.00	4.03	4.20	1.21
	^{13}C	92.2	49.3	79.6	71.2	67.4	16.7
	^{1}H		1.99				
	^{13}C		23.1/				
A′, 4	^{1}H	4.53	4.15	3.92	3.98	4.00	1.17
	^{13}C	102.8	48.7	79.4	70.9	68.3	16.7
	^{1}H		1.95				
	^{13}C		23.1/175.1				
β-A, 1	^{1}H	4.64	3.89	3.80	3.96	3.77	1.26
	^{13}C	95.9	52.8	82.5	70.6	71.7	16.7
	^{1}H		1.99				
	^{13}C		23.1				
A, 4	^{1}H	4.98	3.93	3.83	3.96	3.76	1.28
	^{13}C	100.5	51.6	82.4	70.6	71.9	16.7
	^{1}H		1.95				
	^{13}C		23.1/175.1				
B, 1	^{1}H	5.00/4.96	4.45	4.06	3.85	3.87	
	^{13}C	100.7	51.9	55.9	76.3	78.0	176.2
	^{1}H		2.08	2.24			
	^{13}C		176.5	167.3			
B, 4	^{1}H	4.98	4.43	4.06	3.79	3.87	
	^{13}C	100.5	52.1	55.9	77.3	77.9	176.0
	^{1}H		2.09				
	^{13}C		23.3/176.9				
B′, 4	^{1}H	4.96	4.43	4.06	3.83	3.88	
	^{13}C	100.6	52.1	55.9	76.5	78.0	176.0
	^{1}H		2.09				
	^{13}C		23.3/176.9				
C, 1	^{1}H	4.71	4.50	3.92	3.82	3.82	
	^{13}C	99.7	50.5	57.3	67.1	79.3	176.2
	^{1}H		2.05	2.20			
	^{13}C		176.1	167.1			
C, 4	^{1}H	4.69	4.41	4.11	4.05	3.87	
	^{13}C	100.1	51.2	57.9	71.6	80.0	176.0
	^{1}H		2.06				
	^{13}C		23.0/176.4				
C′, 4	^{1}H	4.70	4.50	3.92	3.82	3.82	
	^{13}C	99.8	50.6	57.4	67.2	79.4	176.0
	^{1}H		2.06				
	^{13}C		23.0/176.4				
X, 4	^{1}H	3.83/3.83	3.85	4.04	4.04	3.95	3.70/4.03
	^{13}C	64.5	83.6	77.8	77.4	82.2	70.4

LPS molecule separated the distal region of the PS from the proximal region still linked to lipid A. This procedure giving information on the linkage between cores and O-chains can be useful in the analysis of many LPSs. A distal GlcN residue of the core, known to be the linkage point of the O-chain structures in other Bordetellae, was transformed into anhydromannose during the process as expected [2]. In *B. pertussis*, this treatment gave rise to a pentasaccharide including the anhydromannose at its proximal end. *B. trematum* yielded a major slower migrating oligosaccharide (compound 4) on TLC. Subsequent reduction, desalting and separation of the products by reverse phase HPLC led to compounds **2**–**4** (Fig. 3).

*3.4. Analysis of compounds **2** and **3***

NMR analysis of compounds **2** and **3** led to the identification of spin systems of 3,5-dideoxy-5-acylaminononulosonic acid and glyceric acid in both of them. These products were reducing monosaccharides. The residue of nonulosonic acid was identified as neuraminic acid by GC–MS of the peracetylated products of acidic methanolysis of both compounds. Glyceric acid was identified by GC–MS as the acetylated methyl

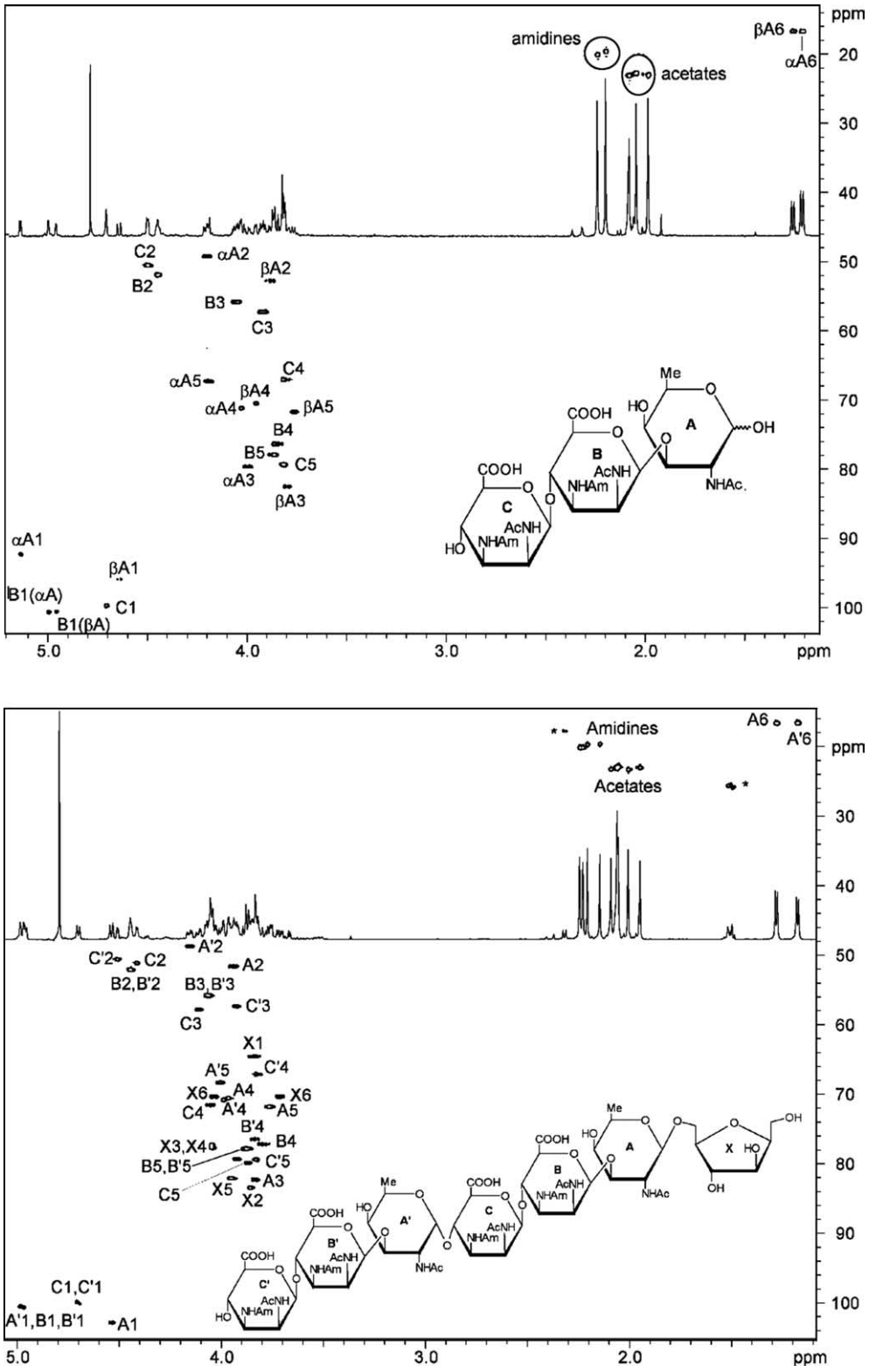


Fig. 1. Two dimensional NMR spectra. Top: NMR spectrum of a *B. trematum* trisaccharide isolated after solvolysis of the polysaccharide region. Bottom: NMR spectrum of a *B. trematum* heptasaccharide isolated from the *B. trematum* lipopolysaccharide after nitrous deamination.

ester and its absolute D-configuration was determined by GC–MS of the acetylated ester with the optically active 2-butanol.

The residue of nonulosonic acid showed coupling constants of $J_{3ax,4}$, $J_{4,5}$ and $J_{5,6}$ of ~11 Hz, $J_{6,7}$ of ~0 Hz, $J_{7,8}$ of 9 Hz, and $J_{8,9ax}$ of 12 Hz. The couplings within the pyranose ring agreed with the D-*glycero*-D-*galacto*-configuration of neuraminic acid. The amino group at C-5 was acylated with glyceric acid, which followed from HMBC correlation between C-1 of glycerate and H-5 on Neu (Table 2). The position of H-3 proton signals at 1.87 (ax) and 2.21 (eq) ppm corresponds to a β-configura-

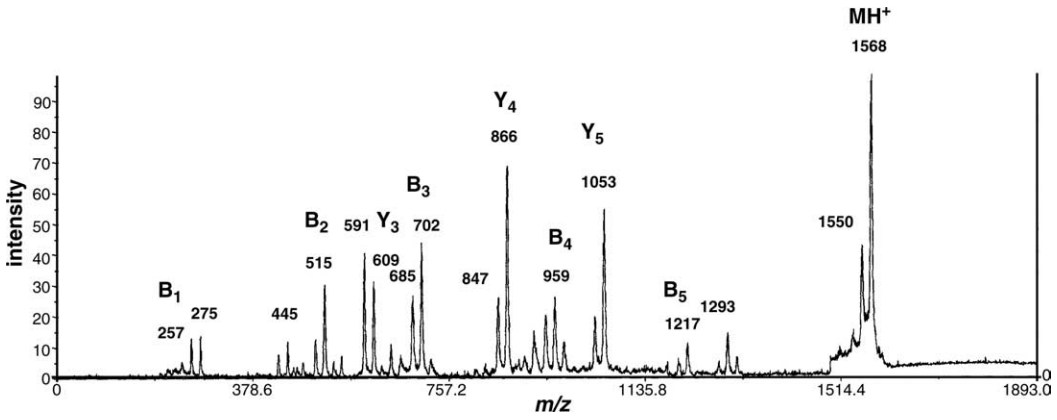
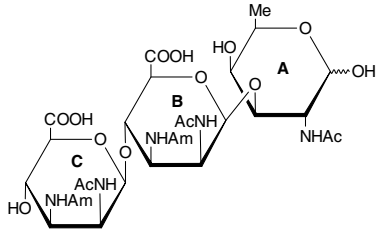
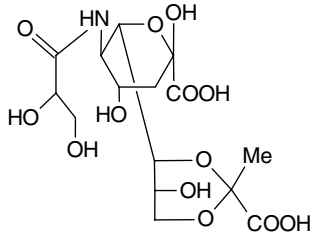


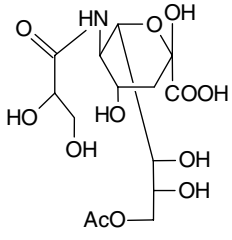
Fig. 2. Post source decay MALDI spectrum of the distal heptasaccharide isolated from the *B. trematum* lipopolysaccharide after nitrous deamination.



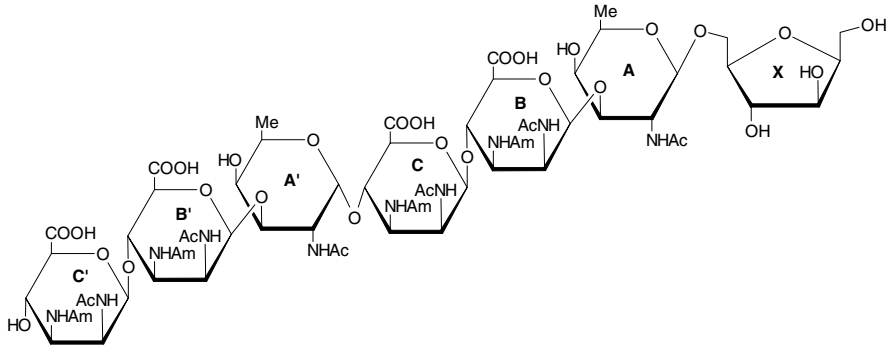
1 trisaccharide



2 7,9-(1-carboxyethylidene)-N-glyceroyl-Neu



3 9-O-acetyl-N-glyceroyl-Neu



4 Heptasaccharide

Fig. 3. Structures of compounds **1**–**4**.

tion (axial carboxyl group), predominant in reducing neuraminic acid. Weak signals of an α-anomer were also visible (H-3eq at 2.72 ppm).

Spectra of compound **2** contained additionally signals of a pyruvate group with a characteristic singlet of a methyl group at 1.45 ppm, which gave HMBC correlations to a quarternary

Table 2
NMR data for compounds **2** and **3**

		1	2 (3ax)	3 (3eq)	4	5	6	7	8	9a (ax)	9b (eq)
	^{1}H		1.87	2.21	4.35	3.77	4.50	3.84	3.88	3.52	3.98
	^{13}C	176.9	96.6	39.6	66.5	52.8	67.1	74.7	59.5	66.1	
3, Neu	^{1}H		1.87	2.28	4.16	4.00	4.16	3.61	3.99	4.21	4.39
	^{13}C			39.8	67.2	52.8	70.5	68.8	68.3	66.9	
2, Py	^{1}H			1.45							
	^{13}C	175.2	101.0	24.1							
2, 3, Ga	^{1}H		4.28	3.84							
	^{13}C	174.9	72.8	63.2							

Py, pyruvate; Ga, glyceric acid.

C-2 carbon at 101 ppm and to a carbonyl group at 175.2 ppm (Table 2). C-2 of the pyruvate residue in 2 showed HMBC correlation to H-9eq and weak correlation to H-9ax. We assumed that the pyruvate residue was present as cyclic acetal, as it is usually encountered. The most probable candidate for ring formation is 7-OH. This corroborates with large couplings $J_{8,9ax}$ and $J_{7,8}$ expected for the six membered dioxane ring of 7,9-O-(1-carboxyethylidene)-neuraminic acid. Large values of these coupling constants and the absence of low-field carbon signals, characteristic for five-membered rings, rule out the possibility of a five-membered ring with the participation of H-8. H-7 in this compound is in the axial orientation, and this is probably the reason for the absence of an HMBC correlation to it from pyruvate C-2, analogous to the very weak signal between pyruvate C-2 and H-9ax. The absence of the NOE correlations between the pyruvate methyl group and any other proton corresponds to its equatorial orientation. Ring formation led to the high-field position of the C-8 signal at 59.5 ppm (compare with 70.7 ppm for free neuraminic acid and with 68.3 ppm in compound 3) and low field shift of C-6 and C-7 signals at 66.1 and 74.7 ppm, respectively (71.0 and 68.9 ppm in free neuraminic acid).

Minor compound **3** contained the same *N*-glyceroylneuraminic acid component found in compound 2, no pyruvate and an additional O-acetyl group (CH_3 signals 2.13/20.7 ppm). O-Acetylation occurred at O-9, which led to the low-field shift of H-9 signals to 4.21 and 4.39 ppm.

The finding of neuraminic acid derivatives is interesting in many respects. First, the novel *N*-glycerol derivative of neuraminic acid was found for the first time in natural products in 1998 [16].

Two derivatives of this monosaccharide were detected – 9-O-acetyl- and 7,9-O-(1-carboxyethylidene). 9-O-acetylated sialic acids are often encountered in living tissues and were shown to be tumor-associated antigens. A compound similar to the pyruvylated Neu has been found only once before, in *Rhodococcus equi* serotype 4 capsular polysaccharide [17], with sialic acid-like monosaccharide of different configuration: 7,9-O-(1-carboxyethylidene)-5-acetamido-3,5-dideoxynonulosonic acid with a *lyxo*-configuration of the C4-C6 fragment, having α-anomeric configuration. It was noted that 7,9-O-(1-carboxyethylidene)-nonulosonic acid was released with anhydrous HF without destruction of the acetal ring. The presence of monosaccharides 2 and 3 among the deamination products is probably a result of simple acid hydrolysis, since deamination is carried out in acidic conditions. It is not clear at this stage of the analysis if this component is linked to the LPS structure. It may be present as a polymer or as single residues, glycosylating the LPS of *B. trematum*. Neuraminic acid derivatives have been described as terminal substituents of core lipooligosaccharides like those of *Haemophilus* and *Neisseria*. This peculiarity prevents human serum bactericidal activity [18].

3.5. NMR analysis of compound 4

The structure of oligosaccharide 4 was analyzed using NMR spectroscopy. Spin-systems of six monosaccharides and of one cyclic alditol were identified in the spectra. Interpretation of the spectra led to the conclusion that it represents a dimer of trisaccharide 1, interconnected by an α-linkage of the FucNAc (A′) residue. Another FucNAc residue (A) was connected via a β-linkage to the 2,5-anhydromannitol residue X. The configuration of the 2,5-anhydromannitol residue was determined by comparison of ^{13}C NMR data with the literature [15] and by GC as peracetate after hydrolysis. It is a product of the reaction of a GlcN residue with nitrous acid. The monosaccharide sequence was determined from NOE (correlations between protons C′1-B′4, B′1-A′3, C1-B4, B1-C3, and C1-X6ab) and HMBC (proton to carbon correlations C′1-B′4, B′1-A′3, C1-B4, B1-C3, and C1-X6) data. The distribution of acyl groups was determined from HMBC data in the same way as for the trisaccharide (compound 1) (Fig. 1). All aminogroups at position 3 of diaminomannuronic acid residues were acylated by acetamidino groups, all other aminogroups were acetylated.

3.6. MS analysis of the heptasaccharide (compound 4)

The heptasaccharide structure was confirmed by MS data: MALDI/MS in the positive mode gave 1568 Da and ESI/MS gave 1567.9 (calculated average 1567.4 Da). MALDI-TOF-MS PSD experiments performed on the heptasaccharide (compound 4) (Fig. 2) showed loss of a non-reducing end corresponding to HexNAc3NAmA (B-type, *m*/*z* 257), a loss of two units of the same sugar (B_2: *m*/*z* 515 and Y_2 : *m*/*z* 1053). The consecutive loss of FucNAc gave fragment ions at *m*/*z* 866 Da (Y-type) and at *m*/*z* 702 (B_3), which confirmed the sequence of sugars. The presence of the second trisaccharide was confirmed by sequential fragmentations of the B and Y-types as indicated in the spectrum. The trisaccharide obtained by HF treatment gave similar results (data not shown).

The *B. trematum* O-chain unit structure is another example of the peculiarity of the Bordetella genus. It has already been reported that all the Bordetella LPS structures analyzed have at least one diaminouronic acid residue as a constituent of its terminal trisaccharide or O-chain, if any. The present structure is the sixth example to be described and this might turn out to be a characteristic of the whole genus.

The presence of different anomeric bonds on the two fucosamine residues in the distal heptasaccharide can be explained by the involvement of two different enzyme systems in the linkages of O-chain units to the core residues, i.e.; a ligase for the first unit and a polymerase for the second. This particularity has already been described in *Salmonella enterica* [19] and *Pseudomonas aeruginosa* [20]. However, the presence of a structure with only two O-chain units is unusual. ''Semi-rough'' strains are usually characterized by the presence of just one O-chain unit [1]. Other examples of irregularity of assembly of the polymer backbone have been described [21].

4. Conclusion

The O-chain of the *B. trematum* LPS has been determined to consist of two trisaccharide sub-units differing in the anomery of the sugar at their non-reducing end. The unambiguous attachment of this ''semi-rough'' O-chain to a glucosamine reinforces the idea that the corresponding glucosamine in other Bordetella LPSs also serves this function.

The source of the neuraminic acid derivatives still remains to be determined.

Acknowledgments: We thank Dr. Perry (NRC, Ottawa) for the production of bacteria and for his interest in the project and Dr. D. Karibian for constructive discussions and help with the manuscript. The MRC/CNRS exchange program gave financial support to M.C. for travel expenses.

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